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Nocardia africana sp. nov., a New Pathogen Isolated from Patients with Pulmonary Infections

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Eight actinomycete strains, isolated from 8 out of 400 sputum samples examined, taken from patients with pulmonary diseases at the Chest Unit of Khartoum Teaching Hospital in the Sudan, were provisionally assigned to the genus *Nocardia* according to morphological criteria. These isolates were studied further in order to establish their taxonomic status. They were found to have morphological and chemical properties typical of nocardiae and formed a monophyletic clade in the 16S ribosomal DNA tree together with *Nocardia vaccinii*. The strains showed a unique pattern of phenotypic properties that distinguished them from representatives of recognized *Nocardia* species, including *Nocardia vaccinii*. The strains were considered to merit species status and were designated *Nocardia africana* sp. nov. The findings of the present study are consistent with the view that pulmonary nocardiosis may occur in a substantial proportion of patients who exhibit chronic lung diseases in African countries. It is important, therefore, that clinicians in such countries consider this condition, especially when patients with respiratory infections fail to respond to antitubercular therapy.

The integrated use of genotypic and phenotypic methods promoted a radical reappraisal of nocardial systematics (11, 13). The genus is now well defined and belongs to the mycolic acid group of actinomycetes—that is, to the suborder *Corynebacterineae* (43), which forms a distinct monophyletic line that encompasses the genera *Corynebacterium, Dietzia, Gordonia, Mycobacterium, Nocardia, Rhodococcus, Skermania, Tsukamurella,* and *Williamsia* (4, 12). Members of these taxa can be distinguished by using a combination of biochemical, chemical, and morphological features (13).

The 19 species which currently comprise the genus *Nocardia* (24) form a monophyletic clade that is enveloped by rhodococci, thereby showing that the genus *Rhodococcus* is paraphyletic (12, 26, 36). The taxonomic status of most of these species is supported by a wealth of data, although there is evidence that the species in the genus are undercounted (13, 24, 31, 46). The improved classification of the genus provides a sound framework for the circumscription of additional nocardial species, including ones that may encompass pathogenic strains.

Nocardiae cause a variety of suppurative infections of humans and animals (11, 27, 41). Human infections may be distinguished clinically into cutaneous, subcutaneous, and lymphocutaneous nocardiosis; extrapulmonary nocardiosis; pulmonary nocardiosis; and systemic nocardiosis involving two or more body sites (40). The incidence of such infections is not known, although nocardiosis has been reported in most regions of the world. Nocardial infections of the internal organs in nontropical countries are mainly caused by *Nocardia asteroides, N. farcinica,* and *N. nova;* relatively few are caused by *N. brasiliensis, N. otitidiscaviarum, N. pseudobrasiliensis,* and *N. transvalensis*. There have been isolated reports of pulmonary nocardiosis from tropical countries caused by *N. asteroides, N. brasiliensis, N. farcinica, N. otitidiscaviarum,* and *N. transvalensis* (15, 19, 21, 23, 25, 30, 32–34, 45).

Recent increases in the reported frequency of human nocardial infections can be attributed to the widespread use of immunosuppressive drugs, improved selective isolation procedures, and increased clinical and microbiological awareness. Nevertheless, in some developing countries where other chronic lung diseases, particularly tuberculosis, are prevalent, nocardiae are either missed or misidentified in laboratory specimens (1, 15). This situation is not satisfactory, because identification of clinically significant nocardiae to the species level is important for establishing the spectrum of disease produced by members of each species and for predicting antimicrobial susceptibility (7, 27).

The primary aim of the present study was to clarify the taxonomy of representative actinomycetes isolated from sputum of patients suffering from pulmonary diseases and presumptively assigned to the genus *Nocardia* by morphological criteria. The organisms were the subject of a polyphasic study, which showed that they form the nucleus of a new species of *Nocardia,* for which the name *Nocardia africana* is proposed.

MATERIALS AND METHODS

Source, isolation, initial characterization, maintenance, and cultivation of isolates. Four hundred sputum samples were taken from seriously ill patients with pulmonary diseases at the Chest Unit of the Khartoum Teaching Hospital in the Sudan. Most of the patients had either not responded to treatment with antitubercular drugs or had responded and then relapsed. Following treatment with the digestion-decontamination procedure of Roberts et al. (37), the sputum samples were concentrated by centrifugation, and the resultant preparations were used to inoculate Löwenstein-Jensen (LJ) (17) slopes, which were incu-

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bated at 37°C for 14 days and then used to make smears, which were examined with a standard Ziehl-Neelsen acid-fast stain.

Eight of the LJ slopes supported the growth of small orange filamentous colonies, which were considered to be typical of nocardiae. The isolates, which were designated SD769, SD771, SD779, SD880, SD910, SD914, SD925, and SD1000, were subcultured and maintained on glucose-yeast extract agar (GYEA) slopes (14) at room temperature and as suspensions of mycelial fragments in glycerol (20% [vol/vol]) at −20°C. All of the isolates were studied phenotypically and chemotaxonomically, and four of them, strains SD769, SD880, SD910, and SD925, were chosen for 16S rRNA sequencing analysis. Biomass for the chemosystematic and 16S ribosomal DNA (rDNA) sequencing studies was grown in shake flasks of GYE broth at 30°C for 10 days and then harvested by centrifugation. The biomass for the chemical analyses was washed twice in distilled water and then freeze-dried; that for the sequencing work was washed in NaCl-EDTA (0.1 M EDTA [pH 8.0], 0.1 M NaCl) and stored at −20°C until required.

Phenotypic properties. The eight isolates were examined for a range of phenotypic properties described by Isik et al. (16). The isolates and the type strains of *N. asteroides, N. brasiliensis, N. farcinica, N. nova,* and *N. otitidiscaviarum* were also tested for their ability to grow in brain heart infusion agar (Oxoid) supplemented with either antibiotics or chemical inhibitors and incubated at 37°C for 3 days.

Chemotaxonomy. The isomeric form of diaminopimelic acid (A_{2pm}) was determined by thin-layer chromatography (TLC) of whole-organism hydrolysates following the procedure described by Staneck and Roberts (44). Standard procedures were also used for the extraction and analysis of mycolic acids (28) and whole-organism sugars (39), with the appropriate marker strains used as controls. Isoprenoid quinones were extracted from freeze-dried biomass (50 mg) by using the small-scale procedure described by Minnikin et al. (29). The purified menaquinones were separated by high-performance liquid chromatography with a Pharmacia LKB instrument equipped with a Spherisorb octyldecylsilane column (5 μm), with acetonitrile-isopropanol (75:25 [vol/vol]) as the mobile phase. The menaquinones were detected at 254 nm.

Sequencing of 16S rDNA. Isolation of chromosomal DNA and PCR amplification of 16S rDNA were carried out by the method of Chun and Goodfellow (3). The amplified preparations were separated by gel electrophoresis, purified with Nucleospin extraction kits (Biogen, Ltd.), and sequenced directly with a *Taq* DyeDeoxy Terminator cycle sequencing kit and previously described primers (3). Sequencing gel electrophoresis was carried out, and the nucleotide sequences were obtained automatically by using an Applied Biosystems DNA sequencer (model 373A) and software provided by the manufacturer.

Phylogenetic analyses. The 16S rDNA nucleotide sequences were aligned manually with corresponding sequences of representative *Nocardia* strains retrieved from the DDBJ, EMBL, and GenBank databases by using the AL16S (2) and PHYDIT (J. Chun, unpublished data) programs. Evolutionary trees were inferred according to four treeing algorithms, namely, by the least-squares (9), maximum-likelihood (8), maximum-parsimony (20), and neighbor-joining (38) methods. The PHYLIP suite of programs, version 3.5c (J. Felsenstein, Department of Genetics, University of Washington, Seattle), was used for all of these analyses. Evolution distance matrices for the least-squares and neighbor-joining methods were prepared as described by Jukes and Cantor (18). Bootstrap analyses were used to evaluate the treeing topologies of the neighbor-joining data by performing 1,000 resamplings with the SEQBOOT and CONSENSE programs included in the PHYLIP package.

Nucleotide sequence accession number. The 16S rDNA sequence accession numbers of the strains are shown in Fig. 1.

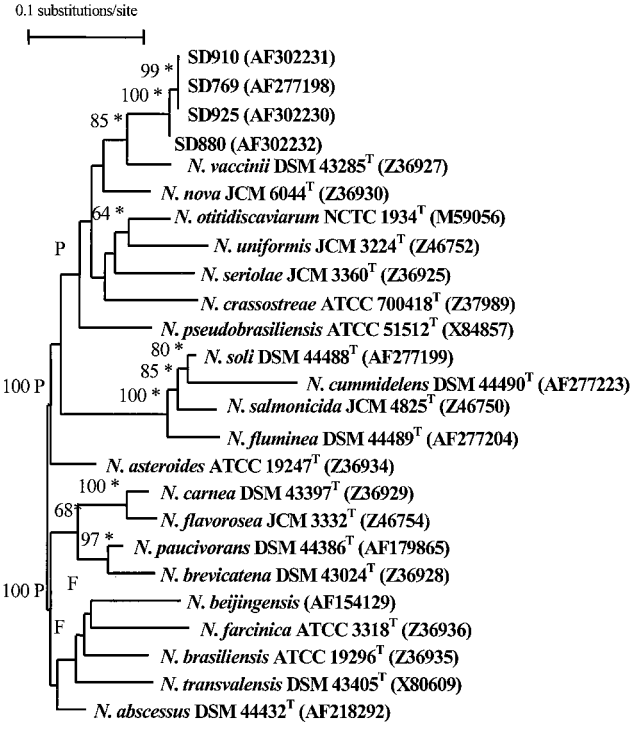

FIG. 1. Neighbor-joining tree based on 16S rDNA sequences showing relationships between clinical isolates and other representatives of the genus *Nocardia*. The asterisks denote the branches that were also recovered by using the other algorithms, namely, the least-squares (9), maximum-likelihood (8), and maximum-parsimony (20) methods. The numbers at the nodes indicate the level of bootstrap support (%) based on a neighbor-joining analysis of 1,000 resampled data sets; only values above 50% are given. The scale bar indicates 0.1 substitution per nucleotide position. T, type strain.

RESULTS AND DISCUSSION

Comparison of the nearly complete 16S rDNA sequences (1,468 of 1,469 nucleotides) of the isolates with corresponding nucleotide sequences of representatives of the suborder *Corynebacterineae* Stackebrandt et al. (43) confirmed that they belong to the genus *Nocardia* (data not shown). Isolates SD769, SD910, and SD925 were found to have identical 16S rDNA sequences. The fourth isolate, strain SD880, shared a 16S rDNA similarity value of 99.9% with each of the other isolates, a value that corresponded to two nucleotide differences. The high 16S rDNA gene sequence similarities to the representatives of the genus *Nocardia* (93.9 to 98.7%) shown by these isolates support their addition to this genus.

The four isolates formed a monophyletic clade with *Nocardia vaccinii* DSM 43285^T in the 16S rDNA tree (Fig. 1). This relationship was supported by using all of the treeing algorithms and by the 85% bootstrap value obtained by the neighbor-joining method. The isolates showed 16S rDNA similarities of between 98.5 and 98.7% with the *N. vaccinii* strain, values which corresponded to between 19 and 21 nucleotide differences. 16S rDNA similarity values within this range have been found between the type strains of *Nocardia brevicatena* and *Nocardia paucivorans* (48), and *Nocardia carnea* and *Nocardia flavorosea* (6), respectively. In each case, the DNA-DNA relatedness values shown by the respective type strains of these species (6, 48) are well below the 70% cutoff point recommended by Wayne et al. (47) for the delineation of genomic species.

The tested strains were found to have phenotypic properties typical of members of the genus *Nocardia* (11, 13). The organisms are aerobic, gram-positive, acid-alcohol-fast actinomycetes that form an extensive branched substrate mycelium, which fragments into irregular, rod-shaped, nonmotile elements and sometimes carries sparse white aerial hyphae, and contains *meso*-A_{2pm}, arabinose, and galactose in whole-organism hydrolysates (wall chemotype IV sensu Lechevalier and Lechevalier)

TABLE 1. Phenotypic properties that distinguish clinical isolates from type strains of validity described *Nocardia* species[a]

Test	Result for: *Nocardia* strains[b]	*N. asteroides* ATCC 19247^T	*N. brasiliensis* ATCC 19296^T	*N. brevicatena* DSM 43024^T	*N. carnea* DSM 43397^T	*N. crassostreae* ATCC 70418^T	*N. cummidelens* DSM 44490^T	*N. farcinica* ATCC 3318^T	*N. flavorosea* JCM 3332^T	*N. fluminea* DSM 44489^T	*N. nova* JCM 6044^T	*N. otitidiscaviarum* NCTC 1934^T	*N. paucivorans* DSM 44386^T	*N. pseudobrasiliensis* ATCC 51512	*N. salmonicida* JCM 4826^T	*N. seriolae* JCM 3360^T	*N. soli* DSM 44488^T	*N. transvalensis* DSM 43405^T	*N. uniformis* JCM 3224^T	*N. vaccinii* DSM 43285^T
Biochemical																				
Esculin hydrolysis	−	+	+	+	+	+	+	+	−	+	+	+	−	+	+	+	+	+	+	+
Arbutin hydrolysis	−	+	+	+	−	ND[c]	+	+	+	+	+	+	ND	−	+	+	+	+	+	−
Nitrate reduction	+	+	+	−	+	ND	+	+	−	+	+	+	ND	−	+	+	+	+	+	+
Urea hydrolysis	−	+	+	−	−	−	+	+	−	−	+	+	+	+	+	−	+	+	+	+
Decomposition of (% [wt/vol]):																				
Adenine (0.4)	−	−	−	−	−	ND	−	−	−	−	−	−	−	+	−	−	−	−	−	−
Casein (1.0)	+	−	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
Elastin (0.3)	−	−	+	−	−	ND	−	−	−	−	−	−	−	+	−	−	−	+	+	−
Hypoxanthine (0.4)	−	−	+	−	−	−	−	−	−	−	−	+	−	+	−	−	−	+	+	−
Tyrosine (0.5)	−	−	+	−	−	−	−	−	−	+	−	−	−	+	+	−	−	−	+	−
Uric acid (0.5)	−	−	−	−	−	ND	−	−	−	−	−	+	ND	+	−	−	−	+	+	−
Xanthine (0.4)	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−	+	−
Growth on carbon source (% [wt/vol]):																				
D-(+)-Mannitol (1.0)	−	+	+	−	+	ND	−	−	+	−	+	+	−	+	+	−	−	+	−	+
α-(L)-Rhamnose (1.0)	−	−	−	+	−	ND	−	+	−	+	−	−	−	−	−	−	+	+	−	+
D-(+)-Sorbitol (1.0)	−	−	−	−	+	ND	−	−	+	−	+	−	−	+	+	−	−	+	−	−
Sodium acetate (0.1)		+	+	+	+	ND	+	+	+	−	+	+	+	+	+	+	+	+	+	−
Sodium citrate (0.1)	−	+	+	−	−	−	−	−	+	+	−	−	−	+	+	+	−	−	−	−
Growth at 45°C	+	−	+	−	−	−	−	+	−	−	−	+	ND	−	−	−	−	−	−	−

[a] Data were derived from this study and from the study by Maldonado et al. (24).
[b] Strains SD769, SD771, SD779, SD880, SD910, SD914, SD925, and SD1000.
[c] ND, not determined.

(22) and mycolic acids that comigrated (R_f value of ca. 0.35) with those extracted from the marker *Nocardia* strains. In addition, the isolates contained predominant amounts of hexahydrogenated menaquinones with eight isoprene units where the two end units were cyclized; this menaquinone is characteristic of members of the genera *Nocardia* and *Skermania* (5, 13). The isolates showed identical biochemical, degradative, and growth profiles (Table 1), which serve to distinguish them from representatives of the recognized species of *Nocardia*, including the type strain of *N. vaccinii*. The isolates can also be distinguished from the type strains of the most clinically significant *Nocardia* species by a range of antibacterial agents (Table 2).

It is evident from the genotypic and phenotypic data that the eight isolates form a new center of taxonomic variation in the genus *Nocardia* (Fig. 1 and Tables 1 and 2). It is therefore proposed that the strains be classified in the genus *Nocardia* as a new species, for which the name *Nocardia africana* sp. nov. is proposed.

Description of *Nocardia africana* sp. nov. (a.fri.ca'na. M.L. fem. adj. *africana*, referring to Africa, the source of the isolates). The bacteria are aerobic, gram-positive, acid-alcohol-fast, nonmotile actinomycetes, which form a branched-substrate mycelium that fragments into irregular rod-shaped elements. Sparse, white aerial hyphae are occasionally formed. Orange, wrinkled colonies are produced on GYEA. Diffusible pigments are not formed. Nitrate is reduced, but esculin, arbutin, and urea are not metabolized. Casein is degraded, but not adenine, elastin, hypoxanthine, tyrosine, uric acid, or xanthine. The organisms grow between 20 and 45°C and in the presence of lysozyme. They utilize sodium propionate as a sole carbon source for energy and growth but do not use mannitol, rhamnose, sorbitol, sodium acetate, or sodium citrate. The organisms are also sensitive to amoxicillin (10 μg ml^{-1}), ampicillin (5 μg ml^{-1}), cephaloridine (100 μg ml^{-1}), doxycycline hydrochloride (10 μg ml^{-1}), erythromycin (100 μg ml^{-1}), lincomycin hydrochloride (100 μg ml^{-1}), neomycin sulfate (100 μg ml^{-1}), novobiocin (10 μg ml^{-1}), penicillin G (100 μg ml^{-1}), spiramycin (5 μg ml^{-1}), and streptomycin sulfate (100 μg ml^{-1}) but resistant to bacitracin (100 μg/ml), cephaloridine (10 μg ml^{-1}), gentamicin sulfate (10 μg/ml^{-1}), kanamycin sulfate (5 μg/ml^{-1}), lincomycin hydrochloride (10 μg/ml^{-1}), lividomycin sulfate (100 μg/ml^{-1}), paromomycin sulfate (100 μg/ml^{-1}), polymyxin B sulfate (50 μg/ml^{-1}), streptomycin sulfate (10 μg/ml^{-1}), and sulfamethoxazole (100 μg/ml^{-1}). Growth occurs in GYEA supplemented with sodium nitrate (0.1%), tetrazolium

TABLE 2. In vitro sensitivities of *N. africana* isolates and type strains of the most clinically significant *Nocardia* species to a range of antibiotic, antibacterial, and chemical inhibitory compounds[a]

Agent	Result for[b]:					
	Nocardia isolates	*N. asteroides* ATCC 19247^T	*N. brasiliensis* ATCC 19296^T	*N. farcinica* ATCC 3318^T	*N. nova* JCM 6044^T	*N. otitidiscaviarum* NCTC 1934^T
Antibiotics and antibacterial agents (μg/ml)						
Amoxicillin						
10	−	−	−	+	−	+
100	−	−	+	−	+/−	−
Ampicillin						
5	−	−	−	+	−	+
50	−	−	−	−	−	+
Bacitracin						
0	+		+	+	+/−	+/−
100	+	+	+	+	+/−	+/−
Cephaloridine						
10	+	−	−	+	+/−	+
100	−	−	−	−	−	+
Erythromycin						
10	+/−	−	+/−	+	−	+
100	−	−	−	−	−	−
Gentamicin sulfate; 10	+	−	+	−	−	+
Kanamycin sulfate						
5	+	−	+	+	+	+
50	+/−	−	−	+	−	−
Lincomycin hydrochloride						
10	+	−	+	+	+	+
100	−	−	−	+	+/−	−
Lividomycin sulfate						
10	+	−	−	−	−	+
100	+	−	−	−	−	+/−
Neomycin sulfate						
10	+/−	−	−	+	−	+
100	−	−	−	−	−	+
Novobiocin; 10	−	−	+	+	+	−
Paromomycin sulfate						
10	+	−	+	−	+	−
100	+	−	−	−	+	−
Penicillin						
10	+/−	−	−	+	−	+
100	−	−	−	+	−	+
Polymyxin B sulfate; 50	+	−	+/−	+	−	+
Spiramycin; 5	−	−	+	+/−	+	+
Streptomycin sulfate						
10	−	−	−	+	−	−
100	+	−	−	+	−	+
Sulfamethoxazole						
10	+	−	+	+	+	+
100	+	−	+	−	+/−	+
Chemical inhibitors (%)						
Pyronin G (0.1)	−	−	−	−	+/−	−
Sodium nitrate (0.1)	+	−	+	+	−	+
Tetrazolium salt (0.1)	+	−	+	−	−	−
Thallous acetate (0.001)	+	−	+	−	−	−

[a] All of the strains grew in the presence of polymyxin B sulfate (5 μg/ml^{-1}) but were completely inhibited by doxycycline hydrochloride (10 μg/ml^{-1}), erythromycin (100 μg/ml^{-1}), gentamicin sulfate (100 μg/ml^{-1}), novobiocin (100 μg/ml^{-1}), spiramycin (50 μg/ml^{-1}), sodium azide (0.01% [wt/vol]), and sodium chloride (7% [wt/vol]).

[b] +, growth equal to or just less than the control plates lacking antibacterial compounds; +/−, little growth compared with control plates; −, no growth.

salt (0.1%), and thallous acetate (0.001%) but not in the presence of pyronin G (0.1%), sodium azide (0.01%), or sodium chloride (7%). The organisms were isolated from the sputum of patients with pulmonary infections.

All of the isolates showed these properties, including the four deposited in culture collections, namely, the type strain SD769 (DSM 44491; NCTC 13181) and isolates SD880 (DSM 44500; NCTC 13182), SD910 (DSM44501; NCTC 13183), and SD925 (DSM 44502; NCTC 13184).

The isolation of nocardiae from sputum taken from patients with respiratory infections is highly indicative of pulmonary nocardiosis (1, 10, 15, 35). In the present study, nocardiae were isolated from sputum of eight patients with a clinical diagnosis of respiratory infection. The patients had symptoms such as fever, productive cough, and weight loss, and in some cases, there was radiological evidence of progressive pulmonary consolidation, which failed to respond to empirical treatment for tuberculosis. These symptoms, together with a failure to respond to antitubercular drugs, are consistent with the view that the patients were suffering from pulmonary nocardiosis, not tuberculosis. These findings are also in line with earlier reports that a substantial proportion of patients that exhibit chronic lung diseases in African countries are suffering from pulmonary nocardiosis (1, 15, 21, 33). The diagnosis of this disease depends on the isolation and identification of the causal organism in an appropriate clinical setting, because clinical, radiological, and histopathological findings are not sufficient for the recognition of pulmonary nocardiosis (40). It is important, therefore, that clinicians in African countries consider this condition, especially when patients with respiratory infections fail to respond to antitubercular therapy.

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